

Irradiation influence on the phenoloxidase pathway and an anti-oxidant defense mechanism in *Spodoptera litura* (Lepidoptera: Noctuidae) and its implication in radio-genetic 'F₁ sterility' and biorational pest suppression tactics

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Abstract

The present study was conducted to appraise the ontogenic radio-sensitivity of a serious tropical pest, *Spodoptera litura* (Fabr.). The molecular responses pertaining to the phenoloxidase (PO) pathway and an anti-oxidant defense mechanism were evaluated in order to understand its implication in pest control at pre-harvest and post-harvest intervals. Irradiation exhibited an inverse relationship with age with respect to impact on developmental and transcriptional responses. Transcript abundance of PO cascade enzymes, prophenoloxidase (*slppo-2*), its activating enzyme (*slppae-1*) and free-radical scavenging enzymes, superoxide dismutase (*slsod*) and catalase (*slcat*) was evaluated upon gamma irradiation alone and the dual-stress of radiation plus microbial challenge. The *slppo-2*, *slppae-1*, *slsod* and *slcat* transcripts were significantly up-regulated in F₁ L6 larvae (6th-instar) resulting from 100 Gy sub-sterilized male adults and unirradiated female moths. The extent of upregulation was relatively higher in comparison with L6 survivors (6th-instar larvae) developed from irradiated neonates (L1) treated with 100 Gy. Upon *Photobacterium* challenge, the transcripts were down-regulated in irradiated L1 suggesting increased larval susceptibility to bacterial infections. Radioresistance increased with the age of the insect, and molecular responses (transcript abundance) of insect defense mechanism were less influenced when older age (F₁ progeny) were irradiated. These findings will help to optimize the gamma dose to be employed in inherited sterility technique for (pre-harvest) pest suppression and (post-harvest) phytosanitation and quarantine, and suggest compatible integration of biorational tactics including nuclear technology.

Keywords: Gamma radiation, F₁ sterility, phenoloxidase pathway, scavenging enzymes, *Spodoptera litura*

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Introduction

The common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is a serious agricultural insect pest in Asia, Australia, New Zealand and Hawaii (IIE, 1993; Zhang, 1994). It has a vast host range of 120 economically

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important plant species, including grains, vegetables, fruits, cotton, jute, tea, tobacco and ornamentals (Venette *et al.*, 2003). The *S. litura* larvae have exhibited high-levels of resistance to insecticides (Ramakrishnan *et al.*, 1984; Armes *et al.*, 1997; Kranthi *et al.*, 2002). There is tremendous pressure from farmers for alternative effective and eco-friendly pre-harvest methods for controlling this pest below economic threshold level.

In this context, the radiation-induced genetic control method known as sterile insect technique (SIT) can be employed as a form of biological control of pest species (Vreysen & Robinson, 2011). This exploits the insect's mate-seeking expertise to introduce genetic abnormalities into the eggs of the wild population. Lepidopteran insects are much more radio-resistant to induction of dominant lethals than insects of other orders such as Diptera (Bauer, 1967; Bakri *et al.*, 2005). The application of a high radiation dose resulting in 100% sterility would reduce the competitiveness of released radio-sterilized male moths (Carpenter *et al.*, 2005; Parker & Mehta, 2007). Therefore, this limitation has led to modification of SIT into F_1 (inherited) sterility technique using a sub-sterilizing dose to the male parent, that is employed for suppression of lepidopteran pests in the next filial generation (North, 1975).

There is a continued growing interest to enhance the efficacy of SIT as a powerful control tactic towards suppression and/or eradication of major Lepidopteran pests (Simmons *et al.*, 2010). The SIT/ F_1 sterility has been studied in many economically important pests of Lepidoptera, including *Cydia pomonella* L., (Proverbs *et al.*, 1978; Bloem *et al.*, 1999a, 1999b, 2001, 2004; Blomefield *et al.*, 2010; Carpenter *et al.*, 2013), *Thaumatotibia leucotreta* (Bloem *et al.*, 2003; Nepgen *et al.*, 2015), *Manduca sexta* (Seth & Reynolds, 1993), *Pectinophora gossypiella* (Staten *et al.*, 1993; Bloem *et al.*, 2000), *Lobesia botrana* (Saour, 2014; Steinitz *et al.*, 2015), *Ephestia kuehniella* (Marec *et al.*, 1999), *Lymantria dispar* L., (Mastro, 1993), *Cactoblastis cactorum* (Carpenter *et al.*, 2001; Hight *et al.*, 2005; Tate *et al.*, 2007), *Phthorimaea operculella* (Saour & Makee, 1997, 2004; Makee & Saour, 2001), *Epiphyas postvittana* (Soopaya *et al.*, 2011; Jang *et al.*, 2012; Follett & Snook, 2012), *Plodia interpunctella* (Ayvaz *et al.*, 2008) and *Plutella xylostella* (Hoa & Tien, 2001).

Certain SIT programs are successfully operational against Lepidopteran pests such as the pink bollworm in the USA (Staten *et al.*, 1993; Bloem *et al.*, 2005; Simmons *et al.*, 2007), the codling moth in western Canada (Dyck *et al.*, 1993; Bloem *et al.*, 2000, 2007a; Vreysen *et al.*, 2010; Knipple, 2013), the false codling moth in South Africa (Carpenter *et al.*, 2007; Hofmeyr *et al.*, 2015), the cactus moth in the USA and Mexico (Hight *et al.*, 2005; Hernández *et al.*, 2007; Bloem *et al.*, 2007b) and the painted apple moth in New Zealand (Wee *et al.*, 2005; Suckling *et al.*, 2007).

Further, this radio-genetic technology (involving F_1 sterility) has also been examined for the management of several noctuid species of Lepidoptera, such as *Helicoverpa armigera* (Ocampo, 2001; Ocampo & De Leon, 2002), *Helicoverpa zea* (Carpenter & Gross, 1993), *Spodoptera frugiperda* (Carpenter *et al.*, 1997, Arthur *et al.*, 2002), *Spodoptera exigua* (Carpenter *et al.*, 1996) and *Trichoplusia ni* (North & Holt, 1969).

We have earlier proposed 100 Gy as an effective sub-sterilizing dose for management of *S. litura* in F_1 sterility program (Seth & Sehgal, 1993; Seth & Sharma, 2001). When a sub-sterilized male moth mates with a normal female (0 Gy), there is a reduction in the production of F_1 progeny, with higher proportion of male offspring that are almost

completely sterilized. The optimal dose of radiation to be used in F_1 sterility involves a trade-off between the level of sterility achieved in treated individuals and physiological damage leading to suppression of competitiveness, relative to wild individuals, both of which would increase with radiation dosage (Suckling *et al.*, 2004a, b; Wee *et al.*, 2005; Kean *et al.*, 2007). The development and growth of F_1 progeny are also likely to be influenced by a carryover effect of the sub-sterilization in male parent. To accomplish this phenomenon, the dose selected should not drastically affect the male fitness and mating performance of the parent and F_1 generation.

The *S. litura* is a serious cut flower pest that requires post-harvest treatment (Dohino *et al.*, 1996). The EPPO has listed *S. litura* as an A1 quarantine pest (OEPP/EPPO, 1979). Several approaches to develop effective post-harvest treatments for the cut flower industry have been tried (Seaton & Joyce, 1988, 1989; Seaton *et al.*, 1989). Disinfestation by gamma irradiation is rapid, effective and can be potentially used. Both floral materials and pest species vary in their sensitivity to radiation (Hansen & Hara, 1994). Therefore, dose selection of gamma radiation is crucial for disinfestation of *S. litura*.

Ultraviolet and gamma irradiation inflict severe alterations in biochemical homeostasis, resulting in oxidative stress (Peng *et al.*, 1986; Datkhile *et al.*, 2009; Wang *et al.*, 2012; Sachdev *et al.*, 2014). Notably, two major pathways, the anti-oxidant defense and phenoloxidase (PO) cascade, are impacted. Detoxification of excessive reactive oxygen species (ROS) is carried out by key anti-oxidant enzymes, mainly superoxide dismutase (SOD), catalase (CAT) and other peroxidases (Felton & Summers, 1995). The radiation-inflicted damaged cells and tissues are repaired by PO cascade zymogens, mainly prophenoloxidase (PPO) and its activating enzyme (PPAE). Upon wounding, bacterial infection and radiation challenge, PPO results in generation of active PO, which leads to melanin deposition at the site of injury (Kanost *et al.*, 2004; Kanost & Gorman, 2008; Jiang *et al.*, 2010).

The present study was conducted to ascertain the transcriptional regulation of PO cascade and free radical scavenging enzymes in irradiated *S. litura* neonates (L1) and adult moth. These results were correlated with the growth and survival of the insect so as to judge the intrinsic quality of P_1 and F_1 insects. These findings will lead to optimization of gamma dose to be employed in inherited sterility technique for pest control and quarantine treatment.

Experimental methods

Insect rearing

A continuous culture of *Spodoptera litura* (Fabricius) was maintained in our insectary at the Department of Zoology, University of Delhi, India. The culture was initially established from larvae and moths collected from infested fields at Indian Agricultural Research Institute (IARI), New Delhi, India. Larvae were reared on a semi-synthetic diet under ambient environmental conditions, $27.0 \pm 1^\circ\text{C}$ temperature, $75 \pm 5\%$ relative humidity and photoperiod of 12:12 h (light:dark) (Seth & Sharma, 2001).

Irradiation of Spodoptera litura neonates and adults

Irradiation was performed at the Radiobiological unit of the Institute of Nuclear Medicine and Allied Sciences (INMAS, Delhi, India). The irradiator was a panoramic ^{60}Co

point source (Gammacell 5000; BRIT, Bhabha Atomic Research Centre, Trombay, India) with a dose rate of ca. 1.91–1.98 KGy h⁻¹ ($\pm 5\%$ Fricke dosimetry).

Two life stages viz. neonates (L1) and freshly eclosed adult moths (0–1 day old) were exposed to gamma radiation. Cohorts of five virgin adult *S. litura* male and female moths were chilled (0–2°C), placed into glass petri dishes and irradiated. Similarly, ca. 50–100 neonates were exposed to radiation. An optimal dosage of 100 Gy was applied based on our previous studies for *S. litura* program (Seth & Sehgal, 1993; Seth & Sharma, 2001). Control neonates and adults were also carried from our laboratory to INMAS radiation facility and were subsequently brought back without any exposure to radiation.

Radio-sensitivity of neonates and F₁ progeny from irradiated male parent in terms of growth, development and survival

The freshly emerged L1 derived from unirradiated (normal) adult moths were irradiated at 100 Gy dose in a group of 25 individuals. Further, male moths sub-sterilized with 100 Gy were crossed with normal females and subsequent F₁ progeny were evaluated for the growth and metamorphic development of the larvae, and correlated with reproductive parameters as described earlier (Seth & Sehgal, 1987, 1993; Seth & Sharma, 2001). Also, the malformation of developed adult was observed in each regimen.

Statistical analysis

Data recorded from the experiments was usually replicated ten times, unless otherwise specified and subjected to analysis of variance (ANOVA). Percentage data was transformed using arcsine \sqrt{x} value before ANOVA, but data shown in tables are back transformations. The *P*-value (≤ 0.05) was considered significant. LSD post-test was performed to determine significant differences among the mean values in different treatments (Snedecor & Cochran, 1989).

Effect of gamma irradiation on transcript abundance of PO pathway and anti-oxidant genes

Larval stages and body tissues

The 100 Gy irradiated neonates (L1) were reared until 6th-instar larvae (L6), and different body tissue was collected from larvae that survived radiation stress. In another regimen, the 100 Gy irradiated male adults were allowed to mate with normal females, and the resulting F₁ progeny that reared until 6th-instar larvae (F₁ L6). Whole body tissue of 3rd-instar larvae viz. naïve L3 (0 Gy), L3 derived from 100 Gy treated L1, and F₁ L3 derived from irradiated male parents crossed with normal females. Hemolymph of 6th-instar larvae viz. naïve L6, L6 derived from 100 Gy treated L1 and F₁ L6 derived from irradiated male parent crossed with normal female, was collected for RNA isolation.

Isolation of hemocytes and whole body tissue

The *S. litura* 6th-instar larvae (L6) were pierced in their prolegs using a sterile needle and hemolymph was collected into pre-chilled anti-coagulant buffer. The mixture was spun at 700×g for at 4°C for 5 min. The supernatant was removed carefully and the pellet containing hemocytes was resuspended in

Trizol reagent (Invitrogen, Carlsbad, CA, USA) and stored at –70°C until further use.

The whole body tissue was rinsed with DEPC-water and then crushed in Trizol reagent using a homogenizer (Sigma Aldrich, MO, USA) and sterile grinder tips.

Total RNA isolation and cDNA synthesis

Both whole body tissue and hemocytes were processed for RNA isolation as per the manufacturer's guidelines. For total RNA isolation from whole body tissue, the number of larvae taken for RNA preparation varied with the size of larvae. Hence, approximately 100 neonates (L1) were pooled per treatment and one larva of 3rd instar (L3) per treatment, were homogenized in 1 ml of Trizol reagent. The lysate was centrifuged at 12,000 × g for 10 min at 4°C to remove the insoluble material. Clear supernatant was transferred to a sterile tube and allowed to stand at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. To the supernatant, 0.2 ml of chloroform was added and mixed by vigorous shaking for 15 s. Phase separation was done by centrifugation at 12,000 × g for 15 min at 4°C. The upper aqueous phase containing RNA was collected in a fresh sterile tube and RNA was precipitated with the addition of 0.5 ml of isopropanol followed by incubation at room temperature for 10 min. The mixture was centrifuged at 12,000 × g for 10 min at 4°C to pellet the RNA. The pellet was washed with 75% ethanol, air-dried and dissolved in DEPC-treated water (Ambion, CA, USA) by heating at 60°C for 10 min.

For total RNA isolation from hemocytes, five larvae of 6th instar (L6) were pooled per treatment to obtain 200 µl of hemolymph and hemocytes were collected as described in the previous section. The pellet containing hemocytes was resuspended in 1 ml of Trizol reagent and total RNA was isolated as described above.

The RNA yield and purity were assessed by two methods: spectrophotometric analysis using NanoDrop 2000 (Thermo-fisher Scientific, DE, USA) and agarose-gel electrophoresis. The amount of RNA was quantified as 5 µg in L1 (100 neonates), 10 µg in L3 (1 larva) and 5 µg in L6 hemocytes (5 larvae were bled). Absorbance ratio was checked and samples with A_{260/280} ratio between 1.8 and 2 were considered superior and subjected to agarose gel analysis. RNA samples, which showed intact bands (28S and 18S) were used in further studies. Genomic DNA (gDNA) contamination from RNA was removed prior to quantitative PCR (qPCR) using RQ1 RNase-free DNase I (Promega, WI, USA). Complete gDNA removal was confirmed by PCR.

cDNA was prepared using 1–5 µg of total RNA of whole body tissue and hemocytes using Superscript-III first strand cDNA synthesis system (Invitrogen, CA, USA) and Oligo (dT) primer. The cDNA was diluted 5-fold and used to test primer efficiencies for Real-time reverse transcription-polymerase chain reaction (RT-PCR).

Bacterial challenge to irradiated larvae (dual stress)

As described above, *S. litura* neonates and adult males were exposed to 100 Gy dose and reared until 6th-instar. The resulting progeny i.e. L6–100 Gy and F₁ L6, were challenged with gram-negative bacterium, *Photothabdus luminiscens* sub spp. *akhurstii* (Strain K-1).

The *P. luminiscens* was grown in 2% LB broth (Luria Bertani) (Difco laboratories, Detroit, MI, USA) at 28°C for

Table 1. Radio-sensitivity of 1st-instar larvae (L1) and adult of *Spodoptera litura* in terms of developmental indices.

Radiation dose ¹	Development period (days) (L1 –Adult)	% Adults eclosion (Malformed ²)	Growth rate ³	Growth index of adult phase ⁴
0 Gy (control)	22.78a ± 0.56	83.9a ± 2.95 (5.6 ± 0.6)	0.5735a ± 0.021	3.68a ± 0.05
100 Gy-L1	27.75b ± 0.48	1.82c ± 0.25 (100 ± 0)	0.1743c ± 0.012	0.065c ± 0.0062
100 Gy-Adult	24.48a ± 0.81	59.8b ± 2.9 (19.5 ± 0.94)	0.3914b ± 0.027	2.2b ± 0.01

¹Freshly emerged larvae from normal eggs and freshly eclosed male adult were irradiated; Irradiated male adult was crossed with normal female and resulting progeny was observed for L1 development and growth upto adult; $n = 10$; number of larvae for each replicate = 25.

²Percent malformed adult out of total adult formation.

³Growth rate calculated for 5th-instar larvae = weight gain/phagoperiod × mean weight.

⁴Growth index = % adult eclosion/total developmental period.

Means ± SE followed by the same letter in a column are not significantly different at $P < 0.05$ (ANOVA followed by LSD post-test); percentage data were arcsine transformed before ANOVA, but data in table are back transformations.

12 h with constant shaking at 180 rpm. The number of the injected bacteria was estimated by plating a known volume of injected suspension (10^7 cells ml^{-1}) on 2% LB-agar plates. An estimation of lethal and sub-lethal dosage was obtained on the basis of the dose at which improper feeding and mortality were caused (data not shown).

The overnight grown bacterial cells were spun down, washed and resuspended in Ringer's solution. Five microlitres of inoculum containing approximately 100 cells of *P. luminescens* were injected directly into the hemolymph of *S. litura* larvae (6th instar, 0–1 day old). The injection was performed by piercing through the proleg using a microinjector (KPS 210, KD scientific, Newhope, PA, USA) with glass syringe (Hamilton, Reno, Nevada, USA). Larvae injected with Ringer's solution alone served as control. Post-injection larvae were kept individually on diet at 25°C. Hemolymph was collected 6 h post-infection, and total RNA was isolated from the hemocytes. Genomic DNA contamination was removed upon treatment with RQ1 DNase (Promega, Madison, WI, USA) at 37°C as per manufacturer's protocol.

Real-time PCR analysis

Primer design and qPCR efficiency. Transcripts of *slppo-2*, *slppae-1*, *slsod* and *slcat* were analyzed by Real-time RT-PCR using SYBR Green RT-PCR kit (Qiagen GmbH, Hilden, Germany) and iCycler™ system (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA sequence of *S. litura* genes encoding *slppo-2*, *slppae-1*, *slsod* and *slcat* were used to design gene-specific primers. The sequences were submitted to Primer3, a web-based tool ensuring that the length of the PCR product was between 250 and 300 bp (Supplementary table 1). In all qPCR experiments, β -actin was used as an internal reference. This has been validated in different insect systems and cell lines viz. *Helicoverpa armigera* (Ahmad *et al.*, 2003; Sivakumar *et al.*, 2007; Rajagopal *et al.*, 2009; Agrawal *et al.*, 2013; Sachdev *et al.*, 2014), *Spodoptera litura* (Rajagopal *et al.*, 2002, 2005; Arora *et al.*, 2009; Singh *et al.*, 2010a; Sree *et al.*, 2010), *Anopheles culicifacies* (Rodrigues *et al.*, 2007; Sharma *et al.*, 2010) and *Sf21* (Singh *et al.*, 2009, 2010b).

qPCR efficiency was evaluated for each gene using the slope of a linear regression model (Pfall, 2001). Standard curves were generated using 5-fold serial dilutions of cDNA ($1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}$) and three technical replicates for each gene. The corresponding qPCR efficiencies (E) were calculated as described by Radonić *et al.* (2004) (Supplementary table S1).

qPCR cycling protocol and data analysis. Each 25 μl reaction mixture contained RNA template (500 ng), 2X Quantitect SYBR Green RT-PCR Master mix, 2 μl (5 picomoles μl^{-1}) of forward and reverse primer and 0.25 μl of Quantitect RT enzyme mix and RNase-free water. Real-time cycler conditions included a preliminary reverse transcription at 48°C for 30 min, an initial activation step at 95°C for 15 min and 40 cycles of denaturation at 94°C, annealing at 52°C and extension at 72°C for 30 s each. The final step included gradual temperature increase from 50 to 94°C at the rate of 1°C 10 s^{-1} to enable melt-curve data collection. A non-template control was run with every assay. Reactions were set-up in triplicates.

The β -actin amplicon was used to normalize each RNA sample. The threshold cycles (C_T) were recorded for *slppo-2*, *slppae-1*, *slsod* and *slcat* and β -actin transcripts for each experiment. The ΔC_T (i.e. difference between C_T of β -actin and C_T of *slppo-2* or *slppae-1* or *slsod* or *slcat*) was determined and the relative transcript was calculated in different treatments using Comparative C_T method using the formula $2^{-\Delta\Delta C_T}$ (Pfall, 2001).

The differences in gene expression level were statistically validated by conducting an unpaired two-tailed *t*-test (Yuan *et al.*, 2006) using GraphPad Prism 5.04 (GraphPad software Inc., California, USA). The P value < 0.05 was considered significant.

Results

Ontogenic stage correlated radio-sensitivity

The somatic growth (in terms of growth rate) and survival of larvae were markedly affected as a consequence of irradiation. Growth rate of 5th-instar larvae (L5) that survived from irradiated neonates (L1) was 0.1743 exhibiting 69.6% reduction with respect to un-irradiated larvae (control). The mortality of control larvae was 9.6%, while it culminated to 87.6% at 100 Gy treatment administered to L1. Adverse effects of radiation were further reflected in the developmental time required by larvae to reach the imaginal stage and on the proportion of adults emerged. Very few treated L1 larvae were able to survive to the adult stage, and they showed retardation in development (about 21.8% prolonged developmental period as compared with the control). Protracted developmental period and high pre-imaginal mortality due to irradiation given to L1 larvae affected the growth index (GI) of the adult phase adversely, exhibiting a 98.2% reduction with respect to control. The other dire effect of the larval irradiation was malformation

in the developed adults. Malformed adults had deformed wings and legs. All adults developed from treated larvae were malformed. Further, upon 100 Gy exposure to male adults and their subsequent mating with normal females, the growth rate of F_1 progeny was reduced to 0.3914 as compared with control (0.5735) (table 1). The developmental time of the F_1 progeny adult stage was slightly affected due to irradiation of the male parent. The GI was reduced by 40.2% with respect to control. Further, 19.6% of eclosed adults exhibited malformations and were not reproductively fit (table 1).

Transcript pattern of PO pathway and anti-oxidant genes upon irradiation

The *S. litura* neonates (L1) and adult males were exposed to gamma irradiation (100 Gy). The larvae that survived radiation stress were reared until 6th-instar (L6) and referred as '100 Gy-L1'. The irradiated adult males were crossed with normal females (unirradiated) and the resulting F_1 progeny (referred as '100 Gy-Adult'), were reared until 6th-instar larvae. The expression pattern of phenoloxidase (PO) pathway and anti-oxidant defense genes mainly, *slppae-1*, *slppo-2*, *slcat* and *slsod*, were analyzed in 3rd-instar (L3) and 6th-instar larvae (L6) to understand age-related differential response upon radiation exposure.

The *slppo-2* and *slppae-1* transcripts significantly downregulated (ca. 10-fold respectively) (P -value < 0.0001) in neonates (L1) that survived direct 100 Gy radiation stress (figs 1a and 2a). This suggests that neonates are highly susceptible to direct gamma radiation exposure.

However, insignificant downregulation of *slppo-2* (1-fold) (P -value = 0.059) and no fold change in *slppae-1* (P -value = 1) transcripts was observed in L3 (whole body tissue) derived from irradiated neonates (figs 1a and 2a). A similar trend was observed in L6 (hemocytes) that survived 100 Gy radiation stress. A 2.4-fold downregulation of *slppo-2* (P -value = 0.002) and no-fold change in *slppae-1* transcript (P -value = 0.703) was noted in L6–100 Gy (hemocytes) as compared with naïve L6 larvae (figs 1b and 2b). This could be attributed to some additional factors present in whole body tissue (such as radioprotectors, anti-oxidants and other secondary metabolites), which are absent in hemocytes.

Both *slppo-2* and *slppae-1* transcripts were significantly downregulated (almost 100-fold and 12-fold) (P -value < 0.0001) in F_1 L3 (derived from 100 Gy-Adult) (Supplementary fig. 1A, B). In contrast, in F_1 L6 hemocytes, significant upregulation of *slppo-2* and *slppae-1* (ca. 10-fold) (P -value < 0.0001) was observed (Supplementary fig. 2A, B). These results suggest that radiosensitivity decreases with the age of the insect. An apparent effect of 100 Gy radiation was observed in *slppo-2* and *slppae-1* transcripts in F_1 larvae as compared with those that were derived from irradiated neonates (Supplementary figs 1 and 2A, B).

The *slsod* transcript was significantly downregulated (almost 10-fold) (P -value = 0.0004) while *slcat* transcript was downregulated ca. 2-fold (P -value = 0.0003) in neonates (L1) that survived direct 100 Gy radiation stress (figs 3a and 4a). Nearly, 2-fold downregulation of *slsod* (P -value < 0.0001) and ca. 1-fold downregulation of *slcat* (P -value < 0.0015) transcripts was observed in L3 (whole body tissue) derived from irradiated neonates (figs 3a and 4a). In contrast, upregulation of ca. 2-fold of *slsod* transcript (P -value < 0.0001) and 3.5-fold of *slcat* transcript (P -value = 0.001) was observed in L6 (hemocytes), which survived 100 Gy radiation stress (figs 3b and 4b).

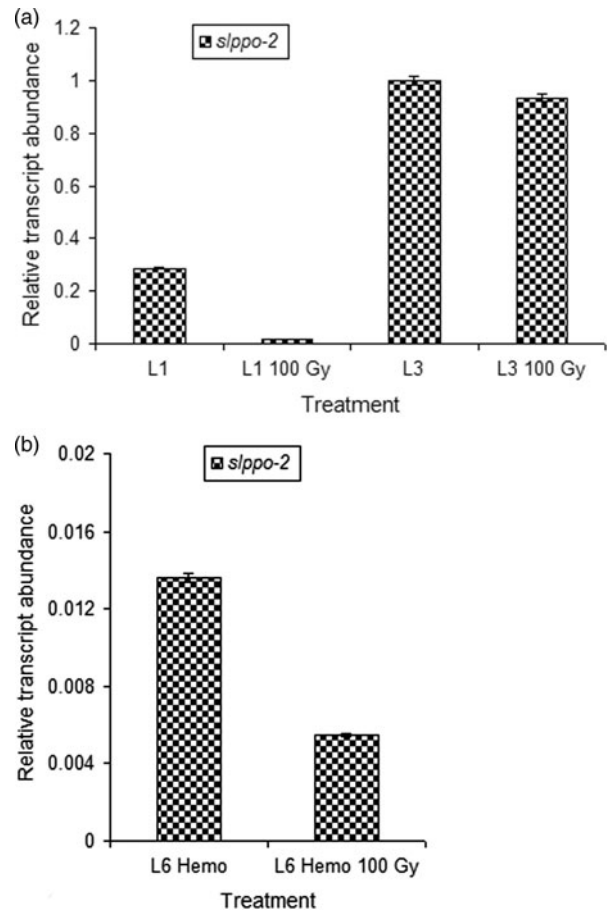


Fig. 1. Transcript profile of *S. litura* prophenoloxidase, *slppo-2*, upon gamma irradiation. *S. litura* neonates (L1) were irradiated at 100 Gy dose and reared until 6th-instar (L6). Total RNA was isolated from (a) whole body tissue of neonates (L1) and 3rd-instar larvae (L3) and (b) hemocytes of 6th-instar larvae (L6). Expression level of *slppo-2* was measured by Real-time RT-PCR using Comparative C_T method and normalized to internal control, β -actin. Error bars indicate the standard error of mean (SEM) for $n=3$ technical replicates. Differences in gene expression were evaluated by performing an unpaired two-tailed t -test (P -value < 0.05).

The *slsod* transcript showed 5-fold downregulation (P -value < 0.0001) in F_1 L3 (derived from 100 Gy-Adult) in contrast to *slcat* transcript which was upregulated (ca. 4-fold) (P -value < 0.0001) (Supplementary fig. 1C, D). In contrast, in F_1 L6 hemocytes, significant upregulation of *slsod* and *slcat* (nearly 5.5-fold and 7-fold) (P -value < 0.0001) was observed (Supplementary fig. 2C, D). The impact of radiation was reduced on *slsod* and *slcat* transcripts when older stage (adults) was irradiated as compared with direct impact on neonates (L1).

Transcript pattern of PO pathway and anti-oxidant genes upon irradiation and bacterial challenge (dual stress)

Neonates of *S. litura* were exposed to 100 Gy (100 Gy-L1), reared to L6 and bacterially challenged with *P. luminiscens*. The F_1 progeny (100 Gy-Adult) derived from irradiated male moths and untreated normal females were reared to L6 and subjected to bacterial challenge. The effect of dual stress was

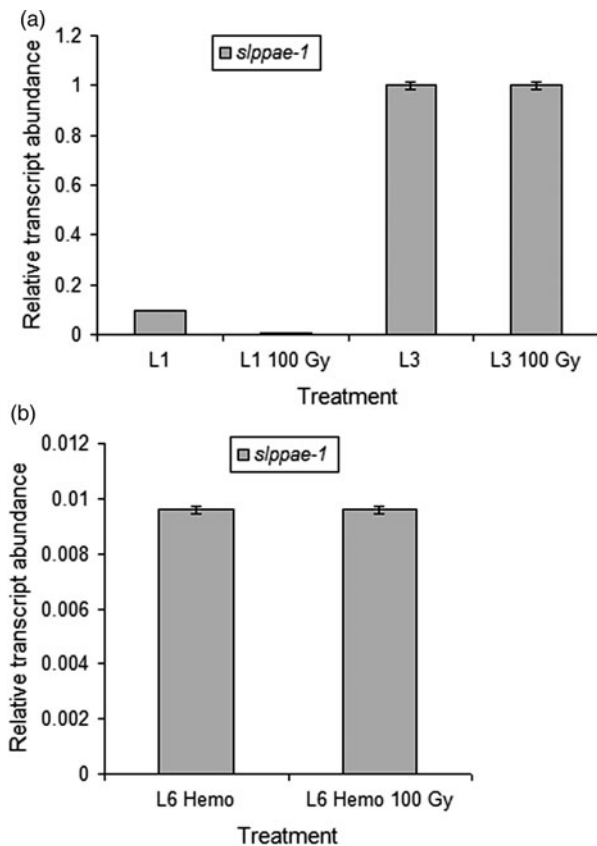


Fig. 2. Transcript profile of *S. litura* prophenoloxidase-activating enzyme, *slppae-1*, upon gamma irradiation. *S. litura* neonates (L1) were irradiated at 100 Gy dose and reared until 6th-instar (L6). Total RNA was isolated from (a) whole body tissue of neonates (L1) and 3rd-instar larvae (L3) and (b) hemocytes of 6th-instar larvae (L6). Expression level of *slppae-1* was measured by Real-time RT-PCR using Comparative C_T method and normalized to internal control, β -actin. Error bars indicate the standard error of mean (SEM) for $n=3$ technical replicates. Differences in gene expression were evaluated by performing an unpaired two-tailed t -test (P -value < 0.05).

examined on transcript abundance of *slppo-2*, *slppae-1*, *slcat* and *slsod* by Real-time PCR.

Bacterial challenge leads to significant up-regulation of *slppo-2* (12-fold; P -value < 0.0001), *slsod* (6-fold; P -value < 0.0001) and *slcat* (3-fold; P -value < 0.0001) transcripts in naïve L6 larvae (untreated) (fig. 5a, c, d). An insignificant upregulation (1-fold; P -value = 0.0039) of *slppae-1* transcript was observed upon bacterial challenge in naïve L6 larvae (fig. 5b).

When L6 (100 Gy-L1) were challenged with *Photobacterium*, nearly 10-fold downregulation of *slppo-2* transcript (P -value < 0.0001) was observed, while no-fold change (P -value = 0.0053) was observed in *slppae-1* transcript level as compared with 'only buffer'-injected L6 larvae derived from 100 Gy-L1 (fig. 5a, b). Both *slsod* and *slcat* transcripts showed insignificant upregulation (ca. 2.5-fold; P -value = 0.0001 and 1-fold; P -value = 0.0010) upon bacterial challenge (fig. 5c, d). These results clearly indicate that neonates are extremely susceptible to direct radiation exposure and their immunity is compromised upon bacterial challenge.

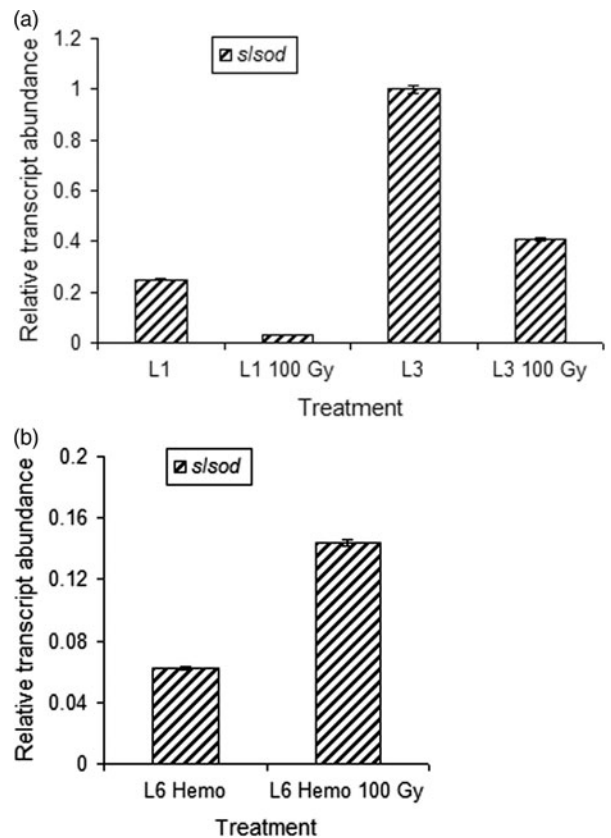


Fig. 3. Transcript profile of *S. litura* superoxide dismutase, *slsod*, upon gamma irradiation. *S. litura* neonates (L1) were irradiated at 100 Gy dose and reared until 6th-instar (L6). Total RNA was isolated from (a) whole body tissue of neonates (L1) and 3rd-instar larvae (L3) and (b) hemocytes of 6th-instar larvae (L6). Expression level of *slsod* was measured by Real-time RT-PCR using Comparative C_T method and normalized to internal control, β -actin. Error bars indicate the standard error of mean (SEM) for $n=3$ technical replicates. Differences in gene expression were evaluated by performing an unpaired two-tailed t -test (P -value < 0.05).

In contrast, when the F_1 L6 hemocytes (100 Gy-Adult) were bacterially challenged, the transcripts of *slppae-1* (ca. 3-fold; P -value < 0.0001), *slsod* (4-fold; P -value < 0.0001), and *slcat* (6-fold; P -value < 0.0001), were significantly upregulated (P -value < 0.0001) as compared with L6 hemocytes (100 Gy-L1), except *slppo-2* (4-fold downregulation; P -value < 0.0001) (fig. 5a-d). These results highlighted that dual stress caused by radiation plus microbial challenge was relatively lesser in F_1 progeny as compared with L6 reared from L1 exposed to direct radiation.

Discussion

The present study was conducted to understand the modulation of radio-sensitivity in (i) *S. litura* larvae exposed to direct radiation and (ii) F_1 progeny of irradiated *S. litura* adult male moths. The consequences of metabolic network specific to immune cascade with respect to larval survival and development have been elaborated. This is the first ever report of correlation of gamma radiation with PO cascade and anti-oxidant defense

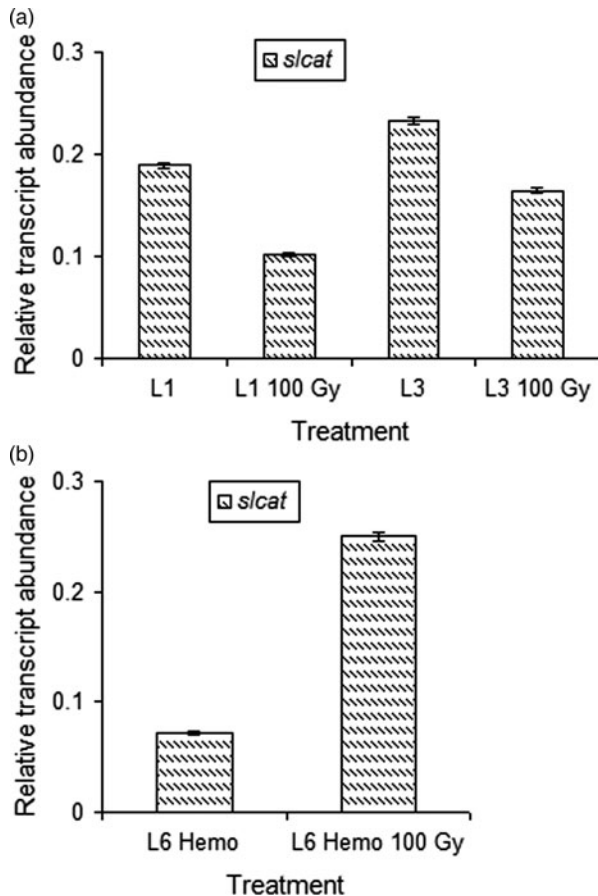


Fig. 4. Transcript profile of *S. litura* catalase, *slcat* upon gamma irradiation. *S. litura* neonates (L1) were irradiated at 100 Gy dose and reared until 6th-instar (L6). Total RNA was isolated from (a) whole body tissue of neonates (L1) and 3rd-instar larvae (L3) and (b) hemocytes of 6th-instar larvae (L6). Expression level of *slcat* was measured by Real-time RT-PCR using Comparative C_T method and normalized to internal control, β -actin. Error bars indicate the standard error of mean (SEM) for $n=3$ technical replicates. Differences in gene expression were evaluated by performing an unpaired two-tailed *t*-test (P -value < 0.05).

genes in *S. litura* at a gamma dose to be employed in radiation-mediated F_1 sterility technique.

Radiosensitivity of neonates and correlation to insect immunity and anti-oxidant defense system

The impact of irradiation was studied in early ontogenic stage, neonates (L1), mainly focusing on insect immunity and an anti-oxidant defense mechanism and correlated with growth and survival of the insect. Nearly identical transcript profiles of PO cascade enzymes, *slppo-2* and *slppae-1* was observed upon L1 irradiation until the final larval instar (L6). The coordinated expression of PPO and PPAAE, co-localized in the insect hemolymph, justify a close metabolic relationship (Arora *et al.*, 2009). Similar debilitating effects of irradiation on PO activity have been demonstrated in Caribbean fruit fly, *Anastrepha suspensa* when 1st-instar larvae were exposed to ≥ 20 Gy (Nation *et al.*, 1995). Irradiation-induced

age-correlated effect on reduced PO activity has been reported in 1st-instar larvae of Mediterranean fruit fly, *Ceratitis capitata* (Mansour & Franz, 1995, 1996) and *Helicoverpa armigera* (Sachdev *et al.*, 2014).

A similar transcript regimen of ROS scavenging enzymes, SOD and CAT, was observed upon L1 radiation and transcripts of *slsod* and *slcat* were downregulated in early instars. Contrastingly, both *slsod* and *slcat* transcripts were upregulated in L6 hemocytes upon 100 Gy-L1 exposure, in agreement to our earlier report on *H. armigera* (Sachdev *et al.*, 2014).

Consistent with the effect of L1 irradiation on PO cascade components and stress responses, the survival of 100 Gy irradiated larvae (L1) to the adult stage was significantly reduced ($\sim 2.8\%$) as compared with control larvae. In a similar study, 90 Gy irradiated neonates of *Amorbia emigratella* (Lepidoptera: Tortricidae) survived to the pupal stage with 9.5% formation, but no adults were formed (Follett, 2008). In another attempt on quarantine control of *Opogona sacchari* (Lepidoptera: Tineidae), irradiation of neonates at 150 Gy resulted in 96% reduction in adult emergence (Hollingsworth & Follett, 2007). The effect of electron beam radiation on survival of cut flower pest, *S. litura* revealed complete mortality in larval stages at >100 Gy (Dohino *et al.*, 1996). Radiation treatment in the larval stage of *S. litura* affected somatic growth and survival (Seth & Sehgal, 1989). Our present study revealed that the developmental period, survival and growth rate, were adversely affected as a consequence of irradiation to *S. litura* larvae. These results are in close agreement with earlier reports on *Heliothis virescens* (Londono *et al.*, 1968), *S. littoralis* (Zaki *et al.*, 1969), *S. exigua* (Zaki *et al.*, 1970) and *H. armigera* (Sachdev *et al.*, 2014).

Molecular responses due to adult irradiation

In order to use adult irradiation in pre-harvest insect control through radio-genetic tactic, the immune and stress responses of F_1 progeny (100 Gy-Adult) were correlated with the growth and reproductive performance. This in turn validated the viability of F_1 progeny at a gamma dose, which could be utilized in F_1 sterility technique. Typically, adult male moths are sub-sterilized by exposure to ionizing radiation, usually a gamma source, causing randomly distributed double-strand breaks in genomic DNA. Chromosomal breaks are the result of both direct energy transfer to DNA and through secondary DNA damage caused by ROS produced during irradiation (von Sonntag, 1987). Anti-oxidant activity may reduce the somatic damage in insects caused by radiation-induced ROS, therefore, maintaining the viability in terms of competitiveness and insect fitness.

The immune cascade and anti-oxidant enzymes are parts of the crucial intrinsic system that might influence the reproductive performance of P_1 and F_1 adults to be employed for radiation-induced inherited sterility in wild population. It is indicated that competitiveness of fully sterile male can be improved by short-term anoxia exposure treatment (López-Martínez & Hahn, 2012) or anti-oxidant rich artificial diet (El-Akhdar *et al.*, 2012). Till date, only few reports have investigated the molecular responses in insects towards irradiation and correlation of these stress-induced responses with biological and physiological activities of irradiated insects (Wang *et al.*, 2012; Sachdev *et al.*, 2014).

The *slppo-2*, *slppae-1*, *slsod* and *slcat* transcripts were significantly upregulated in F_1 L6 hemocytes (100 Gy-Adult) as compared with L6 hemocytes (100 Gy-L1). This clearly suggested

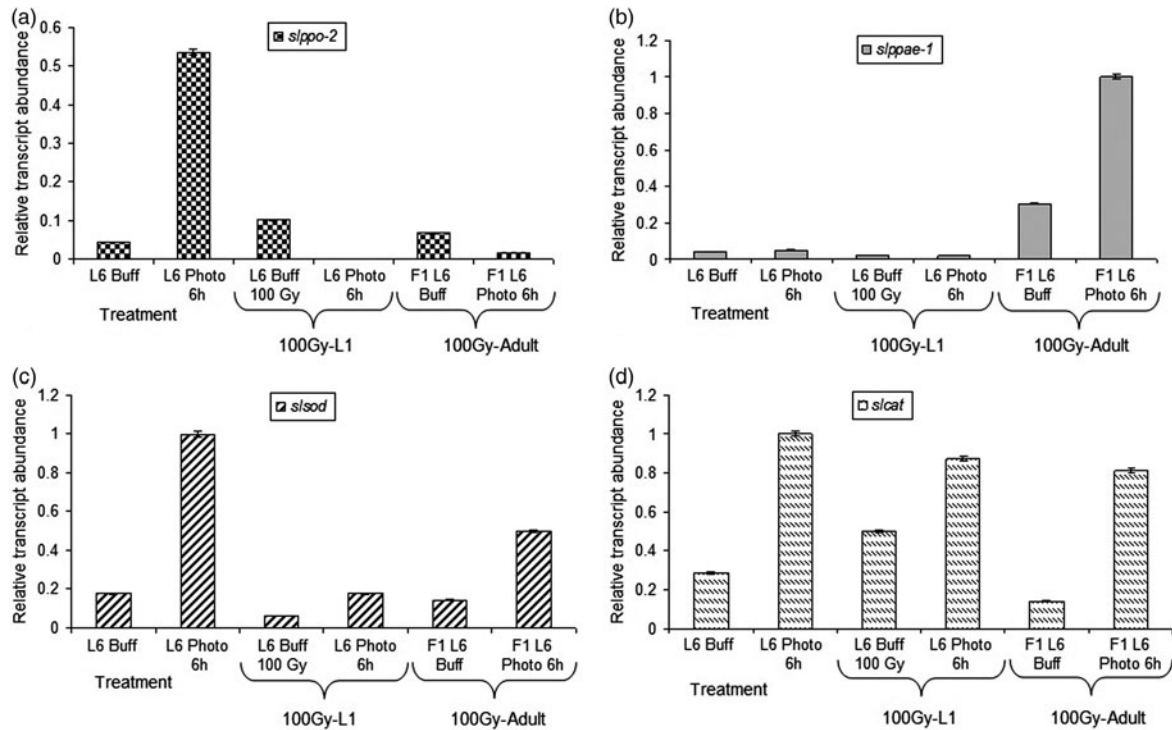


Fig. 5. Temporal induction of PO cascade genes, *slppo-2*, *slppae-1* and anti-oxidant defense genes, *slsod* and *slcat* upon dual stress of gamma radiation and *Photorhabdus*. *S. litura* neonates (L1) were irradiated at 100 Gy and reared until 6th-instar (L6; 100 Gy-L1). Male moths were irradiated at 100 Gy (100 Gy-Adult) and allowed to mate with un-irradiated females. The F_1 progeny was reared until L6 (F_1 L6; 100 Gy-Adult). Approximately, 100 cells of *Photorhabdus* were injected into the hemolymph of larvae [L6; 100 Gy-L1] and [F_1 L6; 100 Gy-Adult]. Hemocytes were isolated at 6 h post-infection. Larvae injected with only buffer (Ringer's solution) served as control. Naive L6 (un-irradiated) challenged with same dose of *Photorhabdus* served as second control. Total RNA was isolated from buffer-injected (L6 Buff), un-irradiated (L6 Photo) and dual-challenged hemocytes (L6; 100 Gy-L1) and (F_1 L6; 100 Gy-Adult). Transcript levels of *slppo-2* (a), *slppae-1* (b), *slsod* (c) and *slcat* (d) were measured by Real-time RT-PCR and were normalized to β -actin expression using Comparative C_T method. Error bars indicate the standard error of mean (SEM) for $n = 3$ technical replicates. Differences in gene expression were evaluated by performing an unpaired two-tailed t -test (P -value < 0.05).

that L1 was more radiosensitive than adult stage and the impact of radiation was relatively reduced when older stage (adult) was exposed. Previous studies have reported that expression level of SOD/CAT is upregulated in response to ROS resulting from exposure of direct sunlight in *Belgica Antarctica* (Lopez-Martinez *et al.*, 2008), UV-B radiation in *Hyphantria cunea* (Kim *et al.*, 2010), UV-A radiation in *H. armigera* (Wang *et al.*, 2012), and gamma rays in *H. armigera* (Sachdev *et al.*, 2014), and *Chironomus ramosus* (Datkhile *et al.*, 2009). The increased expression of SOD/CAT indicated that these enzymes had an important role in protecting cells against oxidative damage thereby maintaining the viability of insects.

The F_1 viability in terms of growth and development of F_1 progeny might reflect the behavioral potency of F_1 adults to be exercised in inherited sterility for pre-harvest pest management (Seth & Sehgal, 1993; Seth & Sharma, 2001). The developmental rate of F_1 adult (100 Gy-Adult) was slower and could be attributed to the protracted development of F_1 progeny due to alteration in hormonal or enzymatic production caused by chromosomal rearrangements (Proshold & Bartell, 1970, 1972) and differential transcriptional regulation of PO pathway genes. The F_1 growth rate and GI (an indicator of somatic damage) showed a decrease as a consequence of irradiation to male parent (sub-sterilized).

Consequences of radiation in immune compromised *S. litura* larvae

Transcriptional analysis of PO cascade and ROS scavenging enzymes was studied in irradiated bacterially-challenged L6 larvae that survived dual stress.

Once inside the larval hemocoel, *Photorhabdus* infection leads to significant upregulation of *slppo-2*, *slsod* and *slcat* transcripts while insignificant upregulation of *slppae-1* was observed in naive L6 larvae. Similarly, infection with non-pathogenic *Escherichia coli* K12 leads to significant upregulation of PPO in *S. litura* (Rajagopal *et al.*, 2005). Bacterial infection with *P. luminiscens* and *E. coli* leads to transcriptional upregulation of anti-bacterial effector genes and pattern recognition proteins in *Manduca sexta* larvae (Felföldi *et al.*, 2011; Eleftherianos *et al.*, 2006a, b, 2007). Activation of proPO-activating proteinase (PAP) has been observed in *M. sexta* fat body upon injection of *E. coli* (Jiang *et al.*, 2003). We have earlier reported that PPAE is triggered upon injury in *S. litura* larvae (Arora *et al.*, 2009). In the present study, we have demonstrated that *slppae-1* transcript is minimally upregulated (ca. 1-fold) upon *Photorhabdus* challenge in naive larvae. This is in consonance with previous findings on three different PAPs isolated from larval hemocytes of *Plutella xylostella* (*px*) (Shi *et al.*, 2014). While transcript abundance of

pxPAPa is significantly increased by *Micrococcus luteus* (G⁺) and *E. coli* (G⁻) but not *Candida albicans*. The *pxPAPb* is only induced upon infection with *M. luteus* and on the other hand, *pxPAP3* transcript was induced by all three microbes (Shi *et al.*, 2014).

Upon dual stress of radiation plus microbes, the *slppo-2* transcript was significantly downregulated in L6 (100 Gy-L1) while *slppae-1*, *slsod* and *slcat* transcripts were insignificantly upregulated. These findings are very close to our previous report on dual stress in *H. armigera*. (Sachdev *et al.*, 2014). A similar transcript regimen was also evaluated in F₁ progeny of irradiated adults. On the contrary to L6 (100 Gy-L1), the *slppae-1*, *slsod* and *slcat* transcripts were upregulated and the F₁ larvae regained their ability to respond to bacterial challenge while *slppo-2* transcript level still remained low. These results not only suggest that neonates are much more radio-sensitive than adult moths but also reflect that the F₁ progeny could counter the somatic damage inherited from sub-sterilized male parent. This could be attributed to the abundance of PO cascade and ROS scavenging enzymes in F₁ progeny, which is positively correlated with the reproductive viability of F₁ insect to be employed in F₁ sterility technique. The overall reproductive performance of P₁ moths (resulting in 50% sterility) and F₁ moths (resulting in 71% sterility) (Seth & Sehgal, 1993; Seth & Sharma, 2001) and the developmental behavior of F₁ insects, supports the release of 100 Gy sub-sterilized males for inherited sterility technique towards suppression of *S. litura* populations. Furthermore, the degree of sterility along with the compromised insect immunity must be equated with the mating competitiveness, quality and quantity of sperm of P₁ and F₁ insects at optimized sub-sterilizing radiation dose (100–130 Gy) for employment in F₁ sterility technique (unpublished data). The present study investigating age-correlated molecular responses to irradiation might help optimize the gamma dose to be employed in phytosanitary treatment of *S. litura* as a quarantine pest, and also in operation of inherited sterility technique for the pest suppression.

Our results provide new insights into the molecular events that occur during immune response in *S. litura*, which are exposed to different microbes in the natural environment.

Radiation-mediated transcript responses of phenoloxidase cascade and anti-oxidant defense genes open up an avenue of coupling microbial control with nuclear technology used in integrated pest management (IPM) at pre-harvest and post-harvest level.

Supplementary material

The supplementary material for this article can be found at <https://doi.org/10.1017/S0007485316000961>

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